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High glucose and diabetes increase the release of [^{3}H]-D-aspartate in retinal cell cultures and in rat retinas

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Abstract

Several evidences suggest that glutamate may be involved in retinal neurodegeneration in diabetic retinopathy (DR). For that reason, we investigated whether high glucose or diabetes affect the accumulation and the release of [^{3}H]-D-aspartate, which was used as a marker of the glutamate transmitter pool. The accumulation of [^{3}H]-D-aspartate did not change in cultured retinal neural cells treated with high glucose (30 mM) for 7 days. However, the release of [^{3}H]-D-aspartate, evoked by 50 mM KCl, significantly increased in retinal cells exposed to high glucose. Mannitol, which was used as an osmotic control, did not cause any significant changes in both accumulation and release of [^{3}H]-D-aspartate. In the retinas, 1 week after the onset of diabetes, both the accumulation and release of [^{3}H]-D-aspartate were unchanged comparing to the retinas of age-matched controls. However, after 4 weeks of diabetes, the accumulation of [^{3}H]-D-aspartate in diabetic retinas decreased and the release of [^{3}H]-D-aspartate increased, compared to age-matched control retinas.

These results suggest that high glucose and diabetes increase the evoked release of D-aspartate in the retina, which may be correlated with the hypothesis of glutamate-induced retinal neurodegeneration in DR.

Keywords: Diabetic retinopathy; Hyperglycemia; Glutamate; Aspartate; Retinal cells

Diabetic retinopathy (DR) is the most frequent cause of blindness among adults in the western countries. This disease has been considered a vascular disease, but recent evidences clearly demonstrate that the neural components of the retina may be also affected. Loss of color vision and contrast sensitivity (Daley et al., 1987; Roy et al., 1986; Sokol et al., 1985) and changes in electroretinograms (Sakai et al., 1995) are early signs of neural retinal dysfunction in diabetic patients. In addition, it was reported an increase in neuronal cell loss and apoptosis in the retina, in diabetic experimental models and humans with diabetes (Abu-El-Asrar et al., 2004; Barber et al., 1998; Martin et al., 2004).

Glutamate is the main excitatory neurotransmitter in the central nervous system, including the retina (Ozawa et al., 1998). However, excessive activation of ionotropic glutamate receptors leads to cell death (Ambrosio et al., 2000; Chen et al., 2001; Ferreira et al., 1998). In some retinal diseases, such as glaucoma and retinitis pigmentosa, toxicity mediated by excessive activation of glutamate receptors seems to play an important role (Vorwerk et al., 1999; Wong, 1994). Regarding DR, several lines of evidence indicate that glutamate may be also involved in retinal degeneration. It was shown that both the metabolism and the concentration of glutamate in the retina are altered in short-term experimental diabetes. Diabetic rat retinas are less able to convert glutamate into glutamine (Kowluru et al., 2001; Lieth et al., 1998). Also, the concentration of glutamate is increased in the vitreous of patients with proliferative DR (Ambati et al., 1997), suggesting that glutamate levels are increased in the retina, possibly without de novo synthesis of glutamate (Lieth et al., 2000). Moreover, the high-affinity L-glutamate/L-aspartate transporter (GLAST) is impaired, in

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retinal Müller cells isolated from streptozotocin-induced diabetic rats, probably due to the oxidation of the glutamate transporter (Li and Puro, 2002).

Since the alterations in the extracellular levels of excitatory amino acids in the retina can be due to changes in their mechanisms of uptake and release, in this study, we investigated the effect of high glucose concentration and diabetes on the accumulation and release of [^{3}H]-D-aspartate, in cultured retinal neural cells and in rat retinas.

1. Experimental procedures

1.1. Materials

Fetal bovine serum was purchased from BioWhittaker (Walkersville, MD, USA) and trypsin (USP grade) was purchased from Invitrogen Corporation (Carlsbad, CA, USA). [^{3}H]-D-aspartate was obtained from Amersham Biosciences (Buckingshire, UK) and Universol scintillation cocktail was purchased from ICN (Irvine, CA, USA). All other reagents were from Sigma Chemical Co. (St. Louis, MO, USA) or from Merck KGaA (Darmstadt, Germany).

1.2. Animals

The animals were treated in agreement with the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in vision and ophthalmic research.

Diabetes was induced with a single intraperitoneal injection of 65 mg/kg streptozotocin (STZ; freshly dissolved in citrate buffer; pH 4.5) in 8 weeks-old male Wistar rats, and was confirmed 2 days later by blood glucose levels exceeding 250 mg/dl.

1.3. Primary cultures of rat retinal neural cells

Retinal cell cultures were obtained from the retinas of 3–5 days-old Wistar rats. Briefly, rat pups were anesthetized by intraperitoneal injection of ketamine (0.05 ml of a 100 mg/ml solution) before decapitation, and the retinas were dissected under sterile conditions, using a light microscope, in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (in mM: 137 NaCl, 5.4 KCl, 0.45 KH_2PO_4, 0.34 Na_2HPO_4, 4 $NaHCO_3$, 5 glucose; pH 7.4). The retinas were digested with 0.1 % trypsin (w/v) for 15 min at 37 °C. After dissociation, the cells were pelleted by centrifugation and resuspended in Eagle's minimum essential medium (MEM) supplemented with 26 mM $NaHCO_3$, 25 mM HEPES, 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). The cells were plated at a density of 2.0×10^6 cells per cm^2 on 24-well plates and 35 mm Petri dishes, coated with poly-D-lysine (0.1 mg/ml), for [^{3}H]-D-aspartate accumulation and release experiments, respectively. The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2/air. After 2 days in culture, the cells were incubated with 25 mM D-glucose (final concentration 30 mM) or with 25 mM D-mannitol (plus 5 mM glucose), which was used as an osmotic control, and maintained for further 7 days. The concentration of glucose in control conditions was 5 mM.

1.4. [^{3}H]-D-aspartate accumulation experiments

Cultured cells were rinsed twice with saline buffer (in mM: 132 NaCl, 4 KCl, 1.4 $MgCl_2$, 1 $CaCl_2$, 1.2 H_3PO_4, 6 glucose, 10 HEPES-Na; pH 7.4), and then incubated with 40 nM D-aspartate and 1 μCi/ml [^{3}H]-D-aspartate in saline buffer, for 45 min, the time necessary to attain the maximal accumulation, at 37 °C. The accumulation of [^{3}H]-D-aspartate was stopped by washing the cells twice with cold (4 °C) saline buffer, and then the cells were lysed with 0.2 M perchloric acid (PCA).

The retinas were rapidly dissected in cold phosphate-buffered saline (PBS; in mM: 137 NaCl, 2.7 KCl, 10 Na_2HPO_4, 1.8 KH_2PO_4; pH 7.4) and rinsed twice in saline buffer (in mM: 118 NaCl, 4.7 KCl, 1.17 $MgSO_4$, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 5.6 glucose; pH 7.4), previously oxygenated. The retinas were then incubated with 40 nM D-aspartate and 2 μCi/ml [^{3}H]-D-aspartate, in oxygenated saline buffer, for 45 min, at 37 °C. The accumulation of [^{3}H]-D-aspartate was stopped by washing the retinas with cold (4 °C) saline buffer, and then the retinas were homogenized in 2 M PCA.

In both cell cultures and retinas, the suspension was centrifuged at 10,000 rpm for 3 min, the supernatant was collected, and the radioactivity was measured in a Packard 2000 Spectrometer provided with dpm correction, using Universol scintillation cocktail.

The results are expressed as the percentage of the accumulated radioactivity relatively to the total radioactivity present in extracellular medium at the beginning of the experiment. In the experiments using intact retinas, the results are expressed per retina, since previous experiments have shown that there are no significant changes in terms of total amount of protein between the various groups.

1.5. [^{3}H]-D-aspartate release experiments

Cultured cells were rinsed in saline buffer (in mM: 132 NaCl, 4 KCl, 1.4 $MgCl_2$, 1 $CaCl_2$, 1.2 H_3PO_4, 6 glucose, 10 HEPES-Na; pH 7.4), and then incubated in saline buffer supplemented with 40 nM D-aspartate and 1 μCi/ml [^{3}H]-D-aspartate, for 45 min, at 37 °C. After loading, the cells were washed three times with saline buffer. The release of [^{3}H]-D-aspartate in cultured cells was measured using a perfusion system, as described previously (Drejer et al., 1987), with some modifications (Santos et al., 1996). Briefly, the Petri dishes were covered with a piece of nylon mesh (pore size of 100 μm), and were placed on a holder slightly inclined (5°). Using two peristaltic pumps, warmed media (37 °C), with the desired composition, was delivered to the dishes with a flow rate of 2 ml/min. Cultured cells were perfused with saline buffer for 15 min. After that period, the fractions were collected every minute with a Gilson FC-204 fraction collector, for 10 min. The cells were stimulated with 50 mM KCl, for 1 min, in saline buffer (*iso*-osmotic substitution of NaCl by KCl to maintain osmolarity). At the end of the experiment, the cell cultures were perfused with 0.2 M HCl for 5 min to collect the remaining intracellular [^{3}H]-D-aspartate.

The retinas were rapidly dissected in cold PBS and then rinsed in cold saline buffer (in mM: 118 NaCl, 4.7 KCl, 1.17 $MgSO_4$, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 5.6 glucose; pH 7.4). The retinas were incubated with 40 nM D-aspartate and 2 μCi/ml [^{3}H]-D-aspartate in the same saline buffer, previously oxygenated, for 45 min, at 37 °C, and then washed three times. The retinas were placed in perfusion chambers, between two pieces of nylon mesh (pore size of 100 μm), and then perfused with saline buffer for 15 min. As previously described for the cell cultures, the fractions were collected every minute and the cells were stimulated for 1 min with 50 mM KCl. At the end of the experiments, the retinas were perfused with 0.2 M PCA for 5 min. To better digest the tissue and collect the residual intracellular [^{3}H]-D-aspartate, 2 M PCA was added to the retinas.

The radioactivity present in each fraction was measured in a Packard 2000 Spectrometer provided with dpm correction, using Universol scintillation cocktail.

The results were expressed as the percentage of radioactivity present in each collected fraction, relatively to the total amount of radioactivity present inside the cells at that time point (fractional release; Figs. 3A, 4A and C); or as the percentage of [^{3}H]-D-aspartate released above basal levels after stimulation, relatively to the total radioactivity accumulated in the cells (Figs. 3B, 4B and D). Total intracellular radioactivity was calculated by adding the radioactivity corresponding to each fraction to the intracellular radioactivity. In the experiments using intact retinas, the results are expressed per retina, since previous experiments have shown that there are no significant changes in terms of total amount of protein between the various groups.

1.6. Statistical analysis

Results are presented as mean ± S.E.M. Statistical significance was determined using one-way ANOVA, and differences between treatments were evaluated using the Dunnet's post-hoc test. Where indicated, statistical analysis was performed using the two-tailed Student's *t*-test.

2. Results

2.1. Effect of high glucose and diabetes on [^{3}H]-D-aspartate accumulation

The effect of high glucose on the accumulation of [^{3}H]-D-aspartate was evaluated in cultured retinal cells. Control cells (5 mM glucose), cells exposed to 30 mM glucose or to 25 mM mannitol (plus 5 mM glucose), which was used as an osmotic control, were incubated with [^{3}H]-D-aspartate for 45 min, the time necessary to attain the maximal uptake (data not shown). The accumulation of [^{3}H]-D-aspartate was similar in the three conditions tested. In control cells, the accumulation of [^{3}H]-D-aspartate was 64.6 ± 5.3% of the total radioactivity added to the incubation medium at the beginning of the experiments, and in cells exposed to high glucose or mannitol the accumulation was 72.1 ± 2.2% or 65.0 ± 3.9% of the total radioactivity, respectively (Fig. 1).

The accumulation of [^{3}H]-D-aspartate was also evaluated in whole retinas of control and diabetic rats (Fig. 2). In the retinas of 1 week diabetic rats, the accumulation of [^{3}H]-D-aspartate (51.9 ± 1.7% of the total radioactivity added to the incubation medium) was similar to the accumulation of [^{3}H]-D-aspartate in the retinas of age-matched control rats (49.9 ± 2.0% of the total radioactivity). However, there was a significant decrease in the accumulation of [^{3}H]-D-aspartate in the retinas of 4 weeks diabetic rats (41.5 ± 1.6% of the total radioactivity), when compared to the retinas of age-matched controls (50.1 ± 0.8% of the total radioactivity). No significant changes were observed between control retinas from both groups (1 and 4 weeks of diabetes), indicating that the differences observed between 1 and 4 weeks diabetic rat retinas were not due to aging.

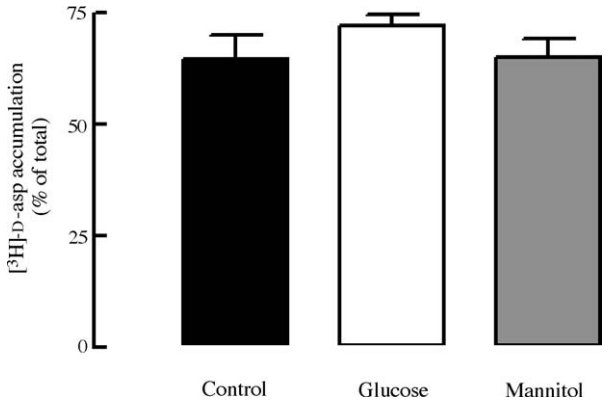

Fig. 1. Effect of high glucose on the accumulation of [^{3}H]-D-aspartate in cultured retinal neural cells. Retinal cells were exposed to high glucose (30 mM) or to mannitol (25 mM) for 7 days. Control cells (black bar), high glucose-treated cells (white bar) and mannitol-treated cells (grey bar) were incubated with 40 nM D-aspartate and [^{3}H]-D-aspartate (1 μCi/ml) for 45 min, at 37 °C. The uptake process was stopped by washing the cells with ice-cold saline buffer. The cells were then homogenized in 0.2 M PCA and centrifuged, and the radioactivity was measured in the supernatants. The results are presented as percentage of total radioactivity added to each well at the beginning of the experiment, and are expressed as the mean ± S.E.M., of at least five independent experiments performed in duplicate.

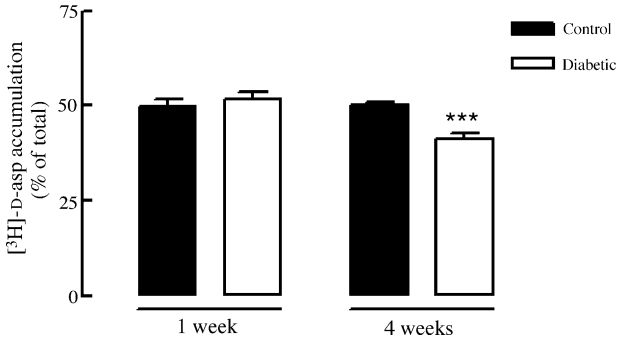

Fig. 2. Effect of diabetes on the accumulation of [^{3}H]-D-aspartate in rat retinas. One and four weeks diabetic rat retinas (white bars) and retinas from age-matched control rats (black bars) were incubated with 40 nM D-aspartate and [^{3}H]-D-aspartate (2 μCi/ml) for 45 min, at 37 °C. The uptake process was stopped by washing the retinas with ice-cold saline buffer. The retinas were then homogenized in 2 M PCA and centrifuged, and the radioactivity was measured in the supernatants. The results are presented as percentage of total radioactivity present in the incubation buffer at the beginning of the experiment, and are expressed as the mean ± S.E.M., of at least six independent experiments. $^{***}p < 0.001$ significantly different of control, *t*-tailed Student's *t*-test.

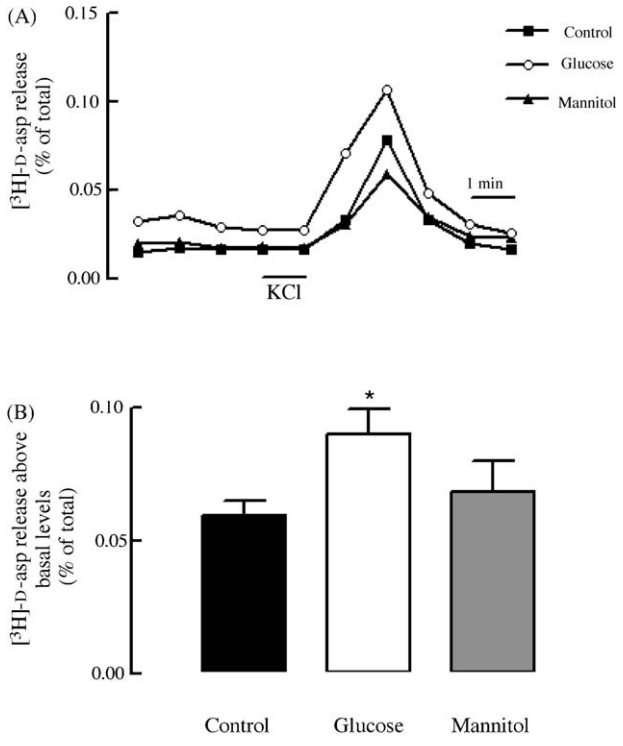

Fig. 3. Effect of high glucose on the release of [^{3}H]-D-aspartate evoked by KCl in retinal cultures. Cells were exposed to high glucose (30 mM) or to mannitol (25 mM) for 7 days. Control cells (black bar), high glucose-treated cells (white bar) and mannitol-treated cells (grey bar) were incubated with 40 nM D-aspartate and [^{3}H]-D-aspartate (1 μCi/ml) for 45 min, at 37 °C. After this period, the cells were kept under continuous perfusion and stimulated with 50 mM KCl for 1 min. (A) Representative graph illustrating the time-course of the efflux of [^{3}H]-D-aspartate. Each point represents the percentage of radioactivity present in each collected fraction, relatively to the total amount of radioactivity present in the cells at that time point. (B) The results represent the percentage of [^{3}H]-D-aspartate released above basal levels, relatively to the total radioactivity accumulated in the cells, and are expressed as mean ± S.E.M., of at least four independent experiments. $^{*}p < 0.05$ significantly different of control, one-way ANOVA followed by Dunnet's post-hoc test.

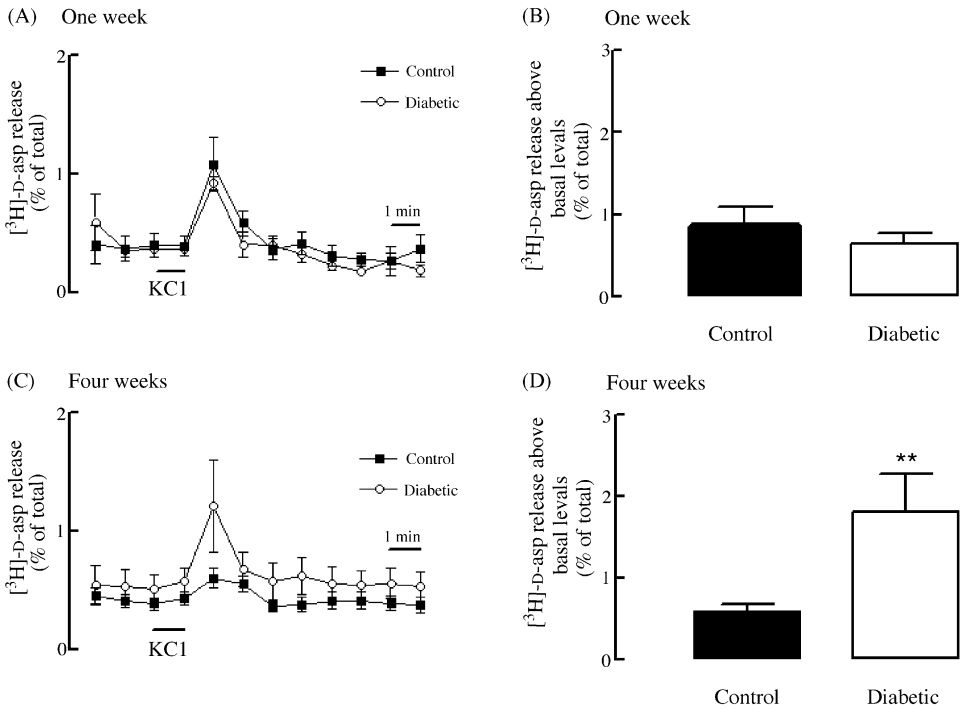

Fig. 4. Effect of diabetes on the release of [^{3}H]-D-aspartate evoked by KCl in rat retinas. The experiments were performed with retinas from 1 week (A and B) and 4 weeks (C and D) diabetic rats (open circles and white bars) and with retinas from age-matched control rats (black squares and black bars). The retinas were incubated with 40 nM D-aspartate and [^{3}H]-D-aspartate (2 μCi/ml) for 45 min, at 37 °C. Retinas were then kept under continuous perfusion and stimulated with 50 mM KCl for 1 min. (A and C) Graphs illustrating the time-course of the efflux of [^{3}H]-D-aspartate. Each point represents the percentage of radioactivity present in each collected fraction, relatively to the total amount of radioactivity present in the retinas at that time point. The results are expressed as mean ± S.E.M. of at least three independent experiments. (B and D) Results represent the percentage of [^{3}H]-D-aspartate released above basal levels upon depolarization, relatively to the total radioactivity accumulated in the retinas, and are presented as mean ± S.E.M., of at least three independent experiments. $^{**}p < 0.01$ significantly different of control, *t*-tailed Student's *t*-test.

2.2. Effect of high glucose and diabetes on [^{3}H]-D-aspartate release

In cultured retinal cells, membrane depolarization with 50 mM KCl (1 min stimulation), induced a transient increase in [^{3}H]-D-aspartate release, as can be observed in Fig. 3A. The release of [^{3}H]-D-aspartate above basal levels, after stimulation, significantly increased in high glucose-treated cells, as compared to control cells. In control cells, the release of [^{3}H]-D-aspartate evoked by KCl was 0.059 ± 0.0058% of the total radioactivity accumulated in the cells (Fig. 3B). In cells exposed to high glucose, the release of [^{3}H]-D-aspartate evoked by KCl increased to 0.090 ± 0.0094% of the total radioactivity, corresponding to 152.5 ± 15.9% of the control. The incubation of retinal cells with mannitol did not affect the release of [^{3}H]-D-aspartate, which was similar to control (0.068 ± 0.0011% of the total radioactivity), indicating that the effect of high glucose was not due to the increase in osmolarity.

As in retinal cell cultures, stimulation of intact retinas with 50 mM KCl, for 1 min, caused a transient increase in the release of [^{3}H]-D-aspartate (see Fig. 4A and C). The release of [^{3}H]-D-aspartate from 1 week diabetic retinas was similar to the release from the retinas of age-matched control rats (Fig. 4A and B). In control retinas, the release of [^{3}H]-D-aspartate above basal levels was 0.86 ± 0.24% of the total radioactivity accumulated in the retinas, and in 1 week diabetic retinas the release was 0.65 ± 0.12% of the total radioactivity.

For longer periods of diabetes (4 weeks), the release of [^{3}H]-D-aspartate from diabetic retinas significantly increased, as compared to the retinas of age-matched controls (Fig. 4C and D). In control retinas, the release of [^{3}H]-D-aspartate evoked by KCl was 0.59 ± 0.08% of the total radioactivity, and in diabetic rat retinas, the release of [^{3}H]-D-aspartate significantly increased to 1.81 ± 0.47% of the total radioactivity, which corresponds to 306.8 ± 79.7% of the control. No significant changes were observed between control retinas from both groups (1 and 4 weeks of diabetes), indicating that the differences observed on the release of [^{3}H]-D-aspartate between 1 and 4 weeks diabetic rat retinas were not due to aging.

3. Discussion

In this study, we demonstrate, for the first time, that elevated glucose and diabetes increase the evoked release of excitatory amino acids from retinal neural cells.

Several evidences point to a role of glutamate in the pathogenesis of DR (Ambati et al., 1997; Kowluru et al., 2001; Lieth et al., 2000; Ng et al., 2004), and we hypothesized that neurodegeneration observed in DR could be due, at least in part, to changes in excitatory amino acids accumulation and/or release. Therefore, we investigated the effect of both high glucose and diabetes on the accumulation and release of [^{3}H]-D-aspartate in retinal cell cultures and in whole retinas, respectively. Retinal cell cultures were exposed to high glucose

for 7 days, because hyperglycemia is considered the primary pathogenic factor in the development of DR.

Since L-glutamate is rapidly metabolized inside the cells, we used [^{3}H]-D-aspartate, which is a non-metabolizing analogue of glutamate, and mimics its behavior. It is transported by glutamate transporters and is released upon cell depolarization (Cousin et al., 1997; Pocock and Nicholls, 1998; Santos et al., 1996).

The accumulation of [^{3}H]-D-aspartate in control and high glucose-treated cells was similar. Also, there were no significant changes in the accumulation of [^{3}H]-D-aspartate in diabetic retinas, 1 week after the onset of diabetes. However, the accumulation of [^{3}H]-D-aspartate significantly decreased after 4 weeks of diabetes, suggesting that the changes in the uptake of excitatory amino acids in the retina do not occur very early in diabetes. This result is in agreement with the work from Li and Puro (Li and Puro, 2002), who reported a decrease in the activity of glutamate transporters, in Müller cells isolated from the retinas of 4 weeks STZ-induced diabetic rats, due to their oxidation. Therefore, a decrease in excitatory amino acids uptake would be expected. Furthermore, decreased glutamate uptake was demonstrated in the brain of Goto-Kakizaki rats, a model of non-insulin-dependent diabetes mellitus (Duarte et al., 2000), but no changes on the expression levels of glutamate transporters were observed in the brain of diabetic animals (Coleman et al., 2004).

The decrease in the uptake of excitatory amino acids could lead to a chronic increase in their levels in the extracellular space and to an overactivation of ionotropic glutamate receptors, which may explain the retinal neuronal dysfunction and possibly neuronal apoptosis that occurs during diabetes. However, it was recently reported that the uptake of glutamate is increased in Müller cells isolated from STZ-induced diabetic rat retinas (Ward et al., 2005). These observations are contradictory to ours, but this discrepancy may be due to the experimental approaches used. While we have measured the accumulation of [^{3}H]-D-aspartate in whole intact retinas, Ward et al. determined the uptake of D-aspartate in retinal cryosections, analyzing the immunoreactivity of D-aspartate in the cell somata of Müller cells. These groups of findings clearly indicate that diabetes elicits alterations in excitatory amino acids transport in the retina, but additional experiments are needed to establish a possible correlation with changes in glutamate transport and cell dysfunction or cell death.

The most important finding in this work is that the evoked release of [^{3}H]-D-aspartate increased in high glucose-treated cells and in diabetic rat retinas (4 weeks of diabetes). These changes were not due to the increase in osmolarity, since mannitol, which was used as osmotic control, did not cause any significant changes. These results may be correlated with previous findings showing that the concentration of glutamate is elevated in the retina in the early course of experimental diabetes (Kowluru et al., 2001; Lieth et al., 1998) and the concentration of glutamate is increased in the vitreous of diabetic patients (Ambati et al., 1997), which may be, at least in part, due to an increase in glutamate release. In addition, glutamine synthetase, the enzyme that converts glutamate into glutamine, appears to be downregulated in diabetic rat retinas (Lieth et al., 2000), resulting in an increase in the concentration of glutamate that can be released through the reversal of the membrane transporters.

Using the same cell culture model, we have observed that the $[Ca^{2+}]_i$ changes evoked by high concentrations of KCl were significantly higher in high glucose-treated cells than in control cells (unpublished observations). This elevation in $[Ca^{2+}]_i$ could explain the increased release of [^{3}H]-D-aspartate in retinal cells cultured in high glucose concentration. The increase in glutamate release upon depolarization might be responsible, at least partially, for increased cell death observed in retinal neurons. Particularly, ganglion cells, which are known to be more susceptible to diabetes (Martin et al., 2004), are enriched in ionotropic glutamate receptors, mostly *N*-methyl-D-aspartate (NMDA) receptors, and overactivation of these receptors may trigger cell apoptosis.

In conclusion, this work shows for the first time, that hyperglycemic conditions increase the release of aspartate in retinal neural cell cultures and in diabetic rat retinas. Thus, this work supports the hypothesis that glutamate may be involved in the pathogenesis of DR. Hyperglycemia may affect the mechanisms of glutamate release, leading to glutamate excitotoxicity, and ultimately to neuronal cell dysfunction and death.

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